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DEVELOPMENT OF A THERAPEUTIC AGENT FOR WOUND-HEALING ENHANCEMENT

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INTRODUCTION

STATEMENT OF PROBLEM

Nonhealing or slow-healing wounds are a major health problem, not only for those persons who suffer from them but also for those who treat them and those who are responsible for the cost of their treatment. Although autologous cellular grafts and reconstructed skin are beneficial in certain instances, such costly specialized and time-consuming procedures are not applicable to all clinical situations. The enhancement of wound healing using pharmaceutical agents can be of great benefit in surgery, burns and dermal ulcers, ophthalmology, and orthopedics, especially for the Navy

The objective of this research is the development of therapeutic agents that can enhance wound healing by stimulating the mitogenesis and chemotaxis of appropriate cells. Platelet-derived growth factor (PDGF) has both these properties and it has been reported that both these properties are located in different domains. Our aim is to identify these active domains. Specifically, we proposed to

- Synthesize a series of peptides of various lengths from the A and B chain sequences of human PDGF. Proteolytic cleavage sites and computer-graphic sequence analysis were considered in selecting the sequence and length of the peptide to be synthesized.
- Test the binding of the peptides to mouse 3T3 cells by radioreceptor assay. These cells have receptors for both PDGF A and B chains.
- Test peptides for mitogenic and chemotactic activity.
- Test potent peptides for wound healing in a mouse model.
- Synthesize smaller fragments of active peptides to determine the minimum sequence required for activity.

BACKGROUND

PDGF is a 32-KD protein heterodimer composed of A and B polypeptide chains linked by disulfide bonds. It is stored in the α-granules of platelets and released when platelets are activated by blood clotting and contact with sites of injury (Deuel and Huang, 1983). It

stimulates specific target cells by binding to cell-surface receptors, thereby mediating a cascade of events that lead to DNA synthesis and cell proliferation. PDGF is a strong mitogen for fibroblasts, smooth muscle cells, and glial cells (Ross and Vogel, 1978; Stiles, 1983). In addition, it is a potent chemotactic factor for neutrophils, monocytes, fibroblasts, and smooth-muscle cells (Deuel *et al.*, 1982; Grotendorst *et al.*, 1981, 1982; Senior *et al.*, 1983; Seppa *et al.*, 1982). Thus, PDGF plays an important role in the migration of inflammatory and connective-tissue cells to sites of inflammation and injury and in the repair or restructuring of injured tissues.

SCIENTIFIC PROGRESS DURING THE PAST YEAR

PEPTIDE SYNTHESIS

During the past year, we synthesized and purified 13 peptides, 4 from PDGF A chain and 10 from PDGF B chain. The sequences of these peptides are shown in Tables 1 and 2. PDGF A (12-28) and (101-125), PDGF B (22-36), (43-57), and (101-117) represent amphipathic helices, in which one face of the helix is occupied preferentially by lipophilic side chains; hydrophilic residues are located at the opposite face. Such a structure provides a stable conformation to the peptide by reacting with membrane through the hydrophobic side of the helix. PDGF B (86-114) was synthesized because this region was reported to show binding affinity (Vogel and Hoppe, 1989). The rest of the peptides were selected on the basis of human leukocyte elastase and cathepsin G cleavage sites. All the peptides were acetylated at the amino terminus and amidated at the carboxy terminus except PDGF B (86-119), to exclude the unnatural free amino and carboxyl groups. Peptides with more than one cysteine in their sequence were cyclized.

Table 1
AMINO ACID SEQUENCES OF THE PEPTIDES SYNTHESIZED FROM PDGF A CHAIN

Peptide	Sequence
PDGF A (12-28)	Ac-T R T V I Y E I P R S Q V D P T S-NH_2
PDGF A (44-51)	Ac-T G C C N T S S-NH_2
PDGF A (44-51) cyclic	Ac-T G C C N T S S-NH_2 (C–C bridge)
PDGF A (101-125)	Ac-D Y R E E D T G R P R E S G K K R K R K R L K P T-NH_2

Table 2
AMINO ACID SEQUENCES OF THE PEPTIDES SYNTHESIZED FROM PDGF B CHAIN

Peptide	Sequence
PDGF B (1-20)	Ac-S L G S L T T A E P A M I A E C K T R T-NH_2
PDGF B (22-36)	Ac-V F E I S R R L I D R T N A N-NH_2
PDGF B (45-52)	Ac-E V Q R C S G C-NH_2
PDGF B (45-52) cyclic	Ac-E V Q R C S G C-NH_2 (C–C)
PDGF B (55-67)	Ac-N R N V Q C R P T Q V Q L-NH_2
PGDF B (86-114)	K A T V T L E D H L A C K C E T V A A A R P V T R S P G G
PDGF B (101-117)	Ac-T V A A A R P V T R S P G G S Q E-NH_2
PDGF B (104-116)	Ac-A A R P V T R S P G G S Q-NH_2
PDGF B (115-128)	Ac-S Q E Q R A K T P Q T R V T-NH_2

EVALUATION OF PEPTIDES

All the peptides mentioned above were tested for cell binding and mitogenesis, and seven were tested for chemotaxis.

EXPERIMENTAL METHODS

PEPTIDE SYNTHESIS

The peptides were synthesized by the solid-phase technique on a Beckman 990C Automated Peptide Synthesizer, starting with commercially available t-BOC amino acid benzhydrylamine (BHA) or methylbenzhydrylamine (MBHA) resin and t-BOC-protected amino acids with the following side-chain protecting groups: O-benzyl esters for aspartic acid and glutamic acid, O-benzyl ethers for serine and threonine, tosyl for arginine and histidine, *p*-methoxybenzyl for cysteine, ortho-chlorobenzyloxycarbonyl for lysine, and 2,6-dichlorobenzyl for tyrosine. All couplings were performed using a 3-mol excess of t-BOC amino acid and dicyclohexyl-carbodiimide (DCC) over the number of millequivalents of amino acid on the resin. Every coupling was repeated, and the repeat coupling was performed as hydroxybenzotriazole (HOBT) active ester. In the case of asparagine and glutamine, both couplings were performed as HOBT active ester. For BOC deprotection, 40% TFA-CH_2Cl_2 containing 10% anisole and 0.1% indole was used. Details of the synthetic cycle are given in Table 3.

Table 3
SCHEDULING FOR PEPTIDE ASSEMBLY ON RESIN

Step	Reagent or Solvent	Time (min)
1.	$CH_2Cl_2 \times 3$	1.5
2.	40% TFA/CH_2Cl_2 prewash	5
3.	40% TFA/CH_2Cl_2	30
4.	$CH_2Cl_2 \times 6$	1.5
5.	5% diisopropylethylamine/$CH_2Cl_2 \times 2$	10
6.	$CH_2Cl_2 \times 3$	1.5
7.	Coupling; 3-fold excess of t-BOC amino acid in CH_2Cl_2:DMF (9:1; v/v); DCC/CH_2Cl_2	120
8.	$CH_2Cl_2 \times 3$	15

ACETYLATION

After the last coupling, the BOC group was removed and the peptide resin was neutralized with 7% diisopropyl ethylamine (DIEA) and washed with CH_2Cl_2 (4×) and DMF. A solution of 500 mg of HOBT per gram of resin in DMF was added followed by 2 mL of acetic anhydride. The mixture was stirred for 1 hr; then the peptide resin was washed thoroughly with DMF, CH_2Cl_2, iPrOH, and CH_2Cl_2 and dried.

CLEAVAGE OF THE PEPTIDE FROM THE RESIN AND PURIFICATION

Peptides were cleaved from the resin by using anhydrous HF in HF-Reaction Apparatus Type II (Peninsula Labs). HF (10 mL/g peptide resin) was distilled into the reaction vessel containing peptide resin plus anisole (1 mL/g resin). If the peptide contained cysteine or methionine, then dimethylsulfide (0.5 mL/g resin) was also added. The mixture was stirred at 4°C for 1 hr. HF was then distilled off completely under vacuum, first at 4°C and then at room temperature. The peptides were separated from the various organic side products by extraction with ether. Peptides were isolated by extraction with 50% acetic acid, then diluted and lyophilized. The crude peptides were purified on high-pressure liquid chromatography (HPLC) using a 20-mm × 50-cm column packed with Vydac 15- to 20-μ C_{18}.

The final peptides were obtained in 20% to 40% yield in >95% purity by HPLC (Figures 1 through 10). Amino acid analysis of all peptides agreed within ±10% of the theoretical value (Table 4).

CYCLIZATION OF PEPTIDES

The cyclic disulfides were formed by air oxidation of a dilute solution of purified peptide in water. The solution pH was adjusted to 8 by adding 50% concentrated NH_4OH in water, and the solution was then shaken slowly on an orbital shaker for 7 days. An Ellman test was then performed to confirm the completion of the disulfide bond formation. The solution was then lyophilized.

CELL BINDING ASSAY (COMPETITION FOR RECEPTOR BINDING)

Balb/c 3T3 cells were set up at 350,000 cells per well in 24-well dishes. The next day, the cells were prepared for the binding assay by washing the monolayer at room temperature 3× with 1 mL Dulbecco's modified Eagle's medium without serum (DMEMo) per well. The cells were next washed once with 1 mL DMEMo containing 0.5% (w/v) BSA (binding buffer) at 4°C.

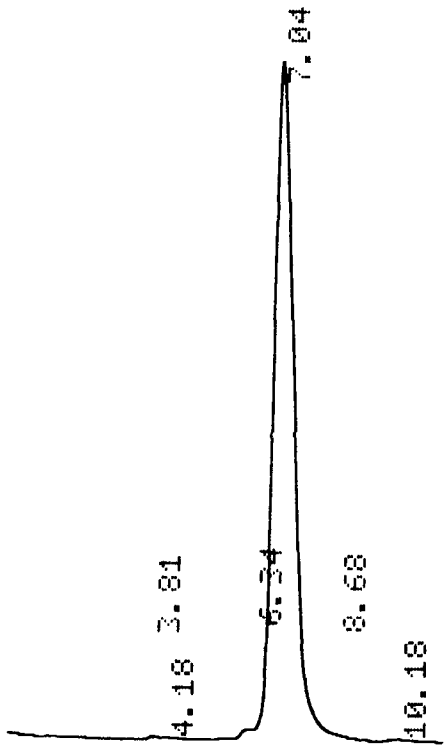

Figure 1. HPLC profile of PDGF A (12-28).
Solvent: Isocratic 23% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

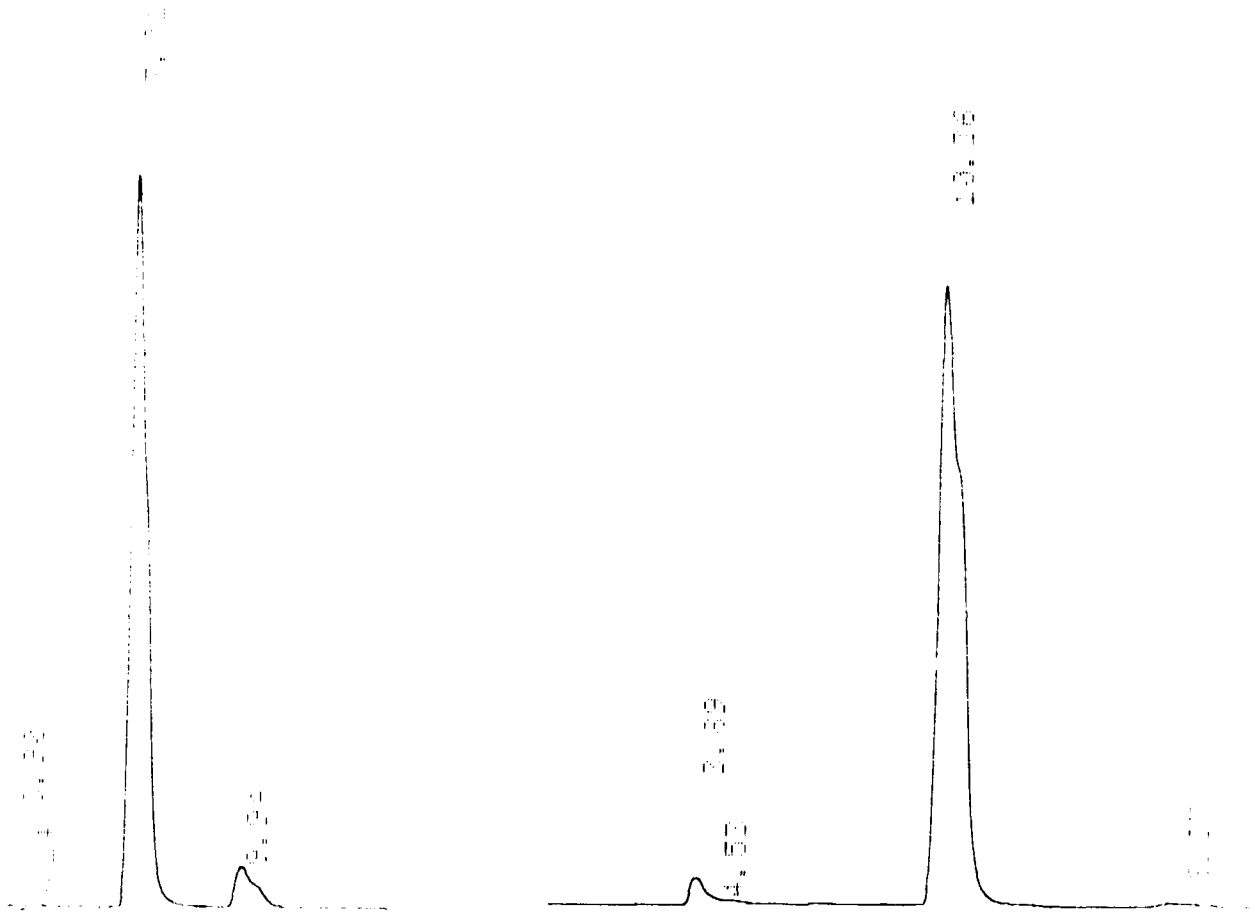

Figure 2. (A) PDGF A (44-51) linear; (B) Cyclic.
Solvent: Isocratic 8% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

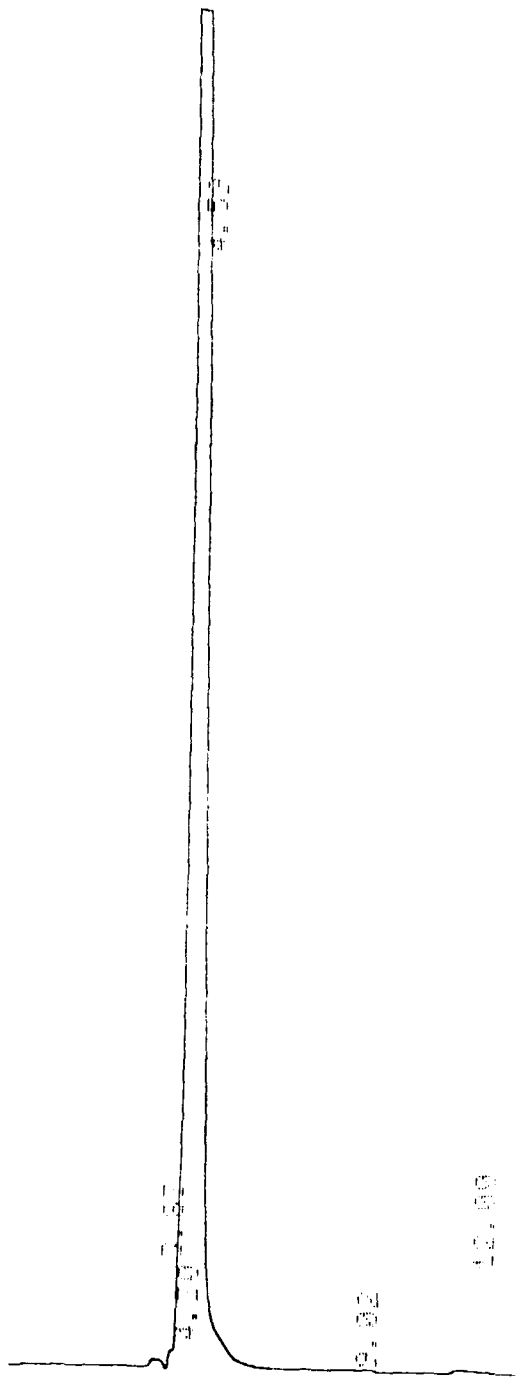

Figure 3. HPLC profile of PDGF A (101-125).
Solvent: Isocratic 18% CH_3CN + 0.1% TFA in H_2O.
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm

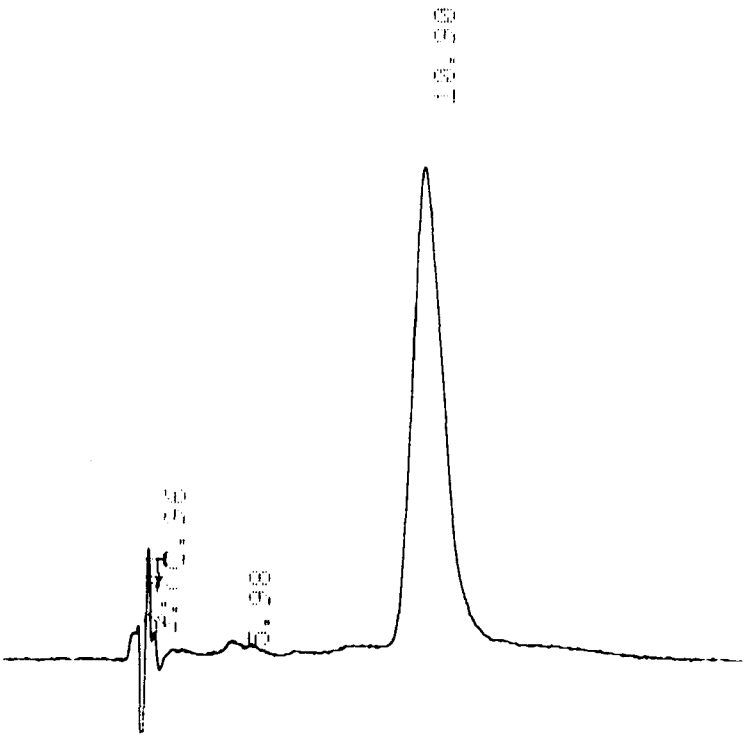

Figure 4. HPLC profile of PDGF B (1-20).
Solvent: Isocratic 25% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

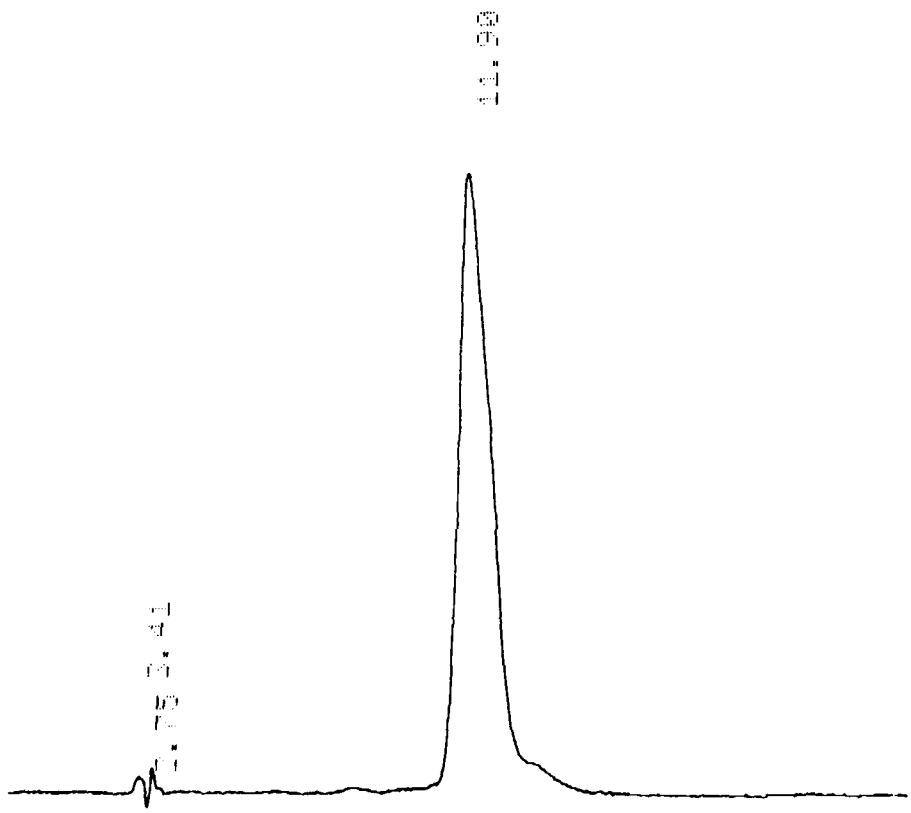

Figure 5. HPLC profile of PDGF B (22-36).
Solvent: Isocratic 30% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

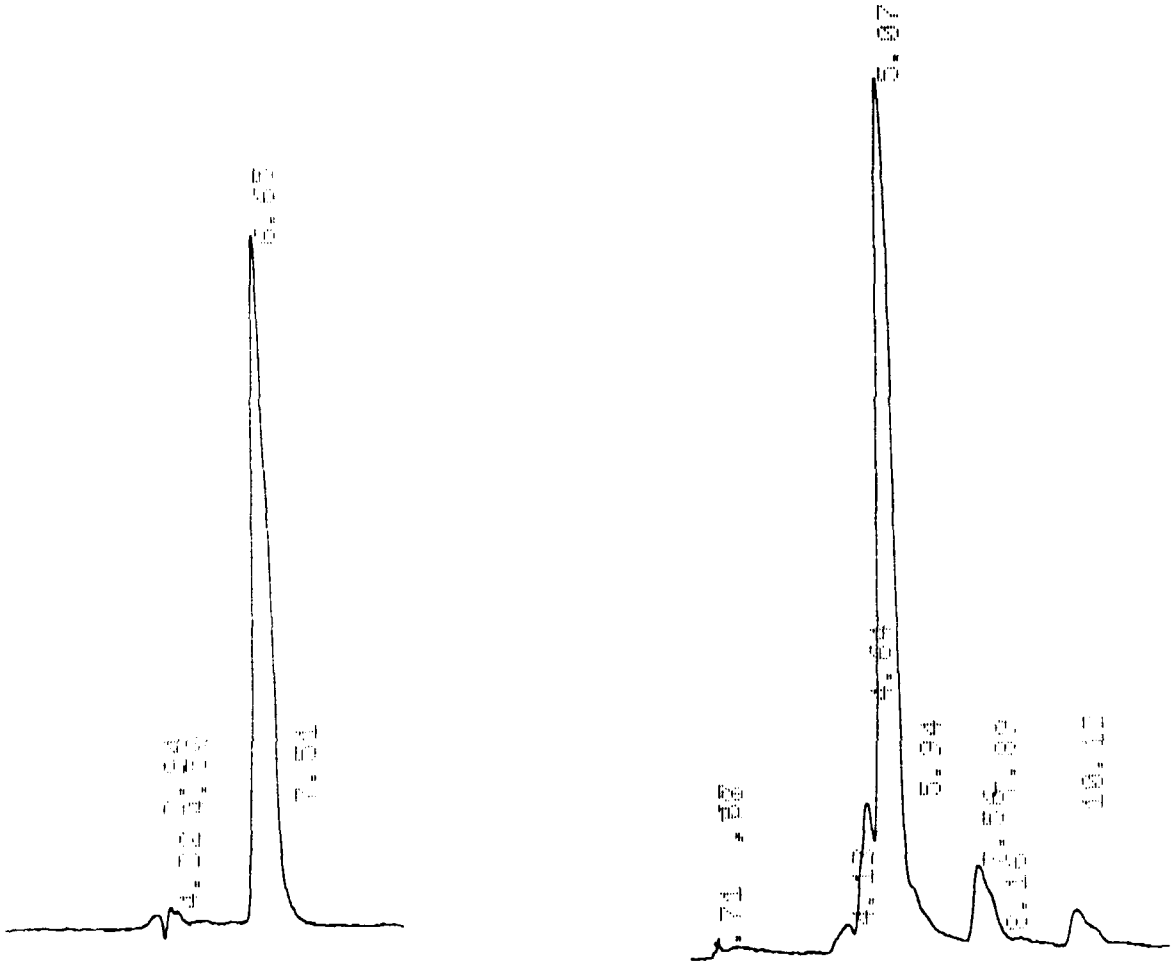

Figure 6. (A) PDGF B (45-52) linear; (B) Cyclic.
Solvent: Isocratic 13% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

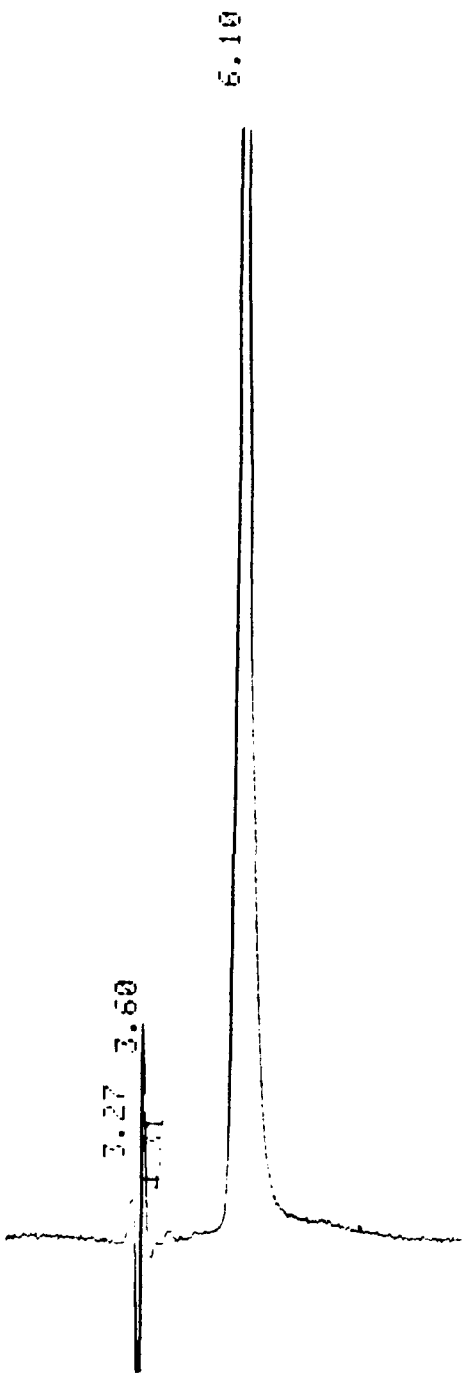

Figure 7. HPLC profile of PDGF B (55-67).
Solvent: Isocratic 18% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

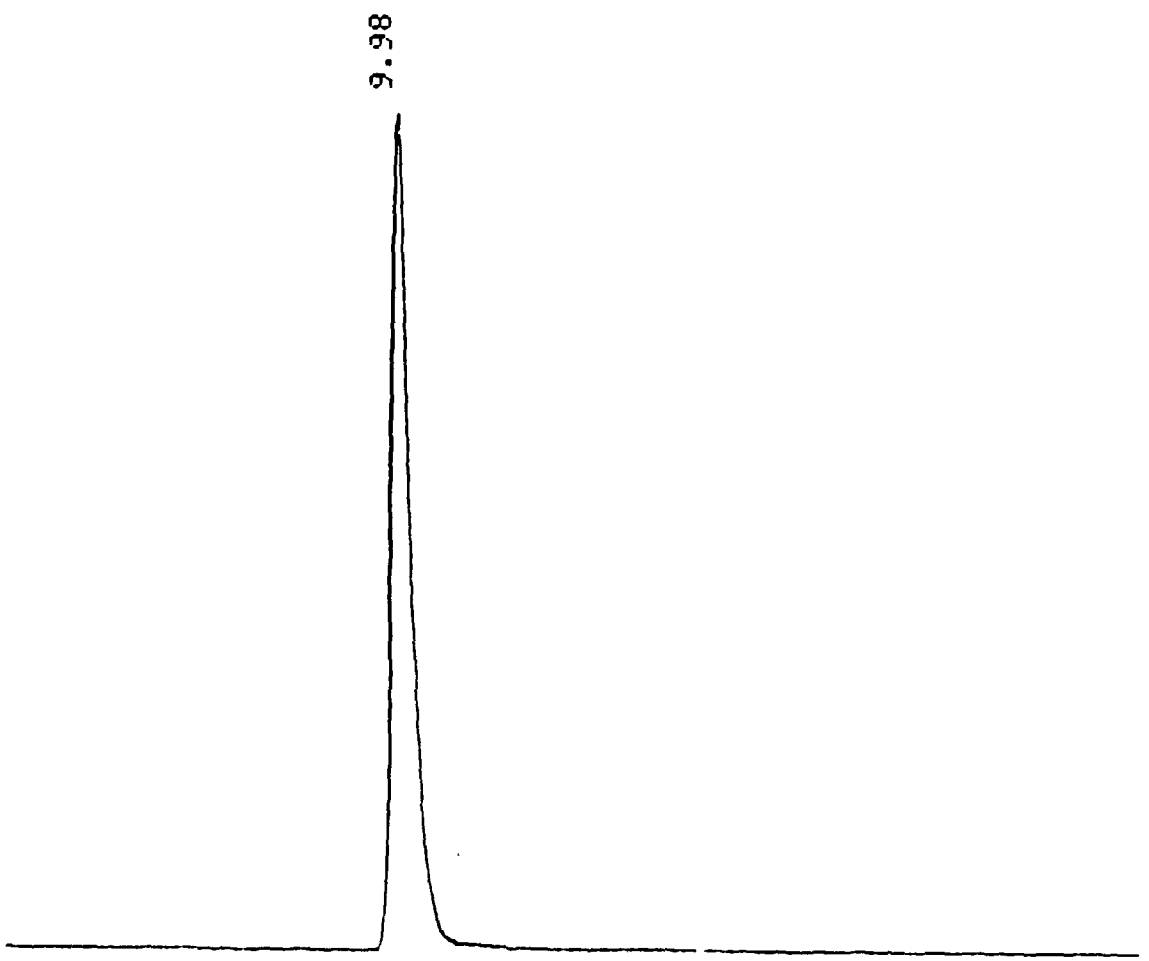

Figure 8. HPLC profile of PDGF B (101-117).
Solvent: Isocratic 23% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

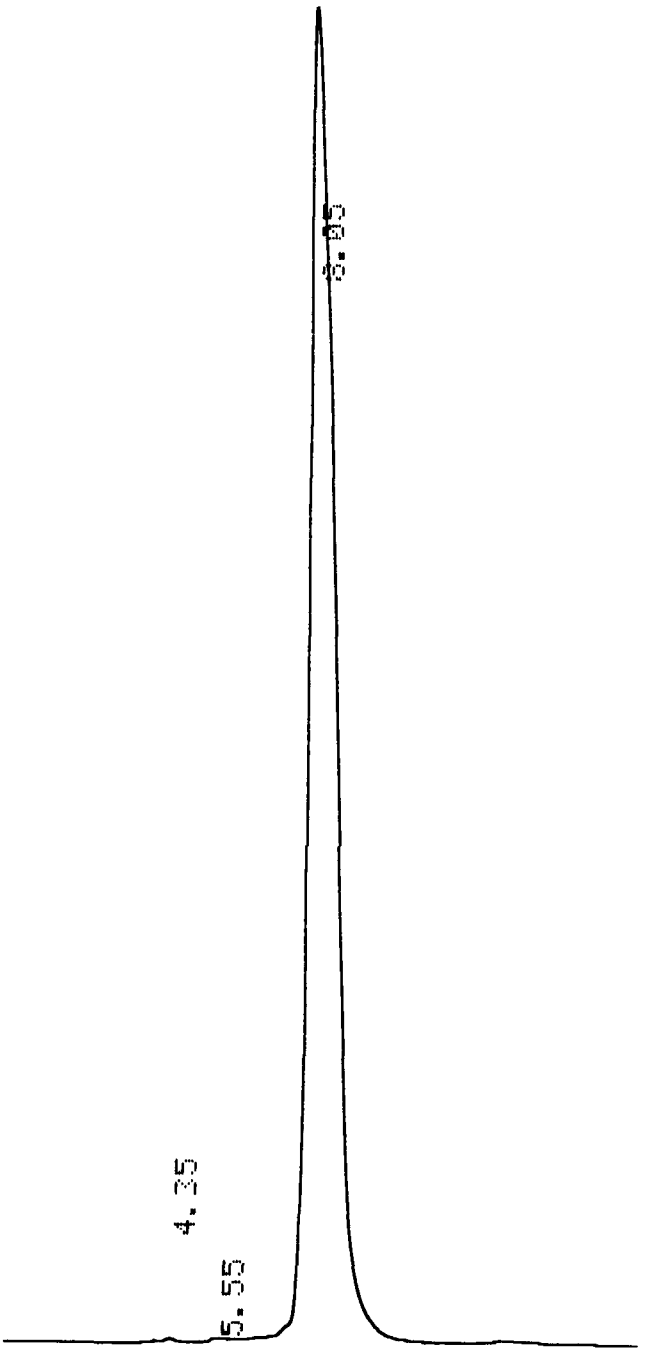

Figure 9. HPLC profile of PDGF B (104-116).
Solvent: Isocratic 10% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

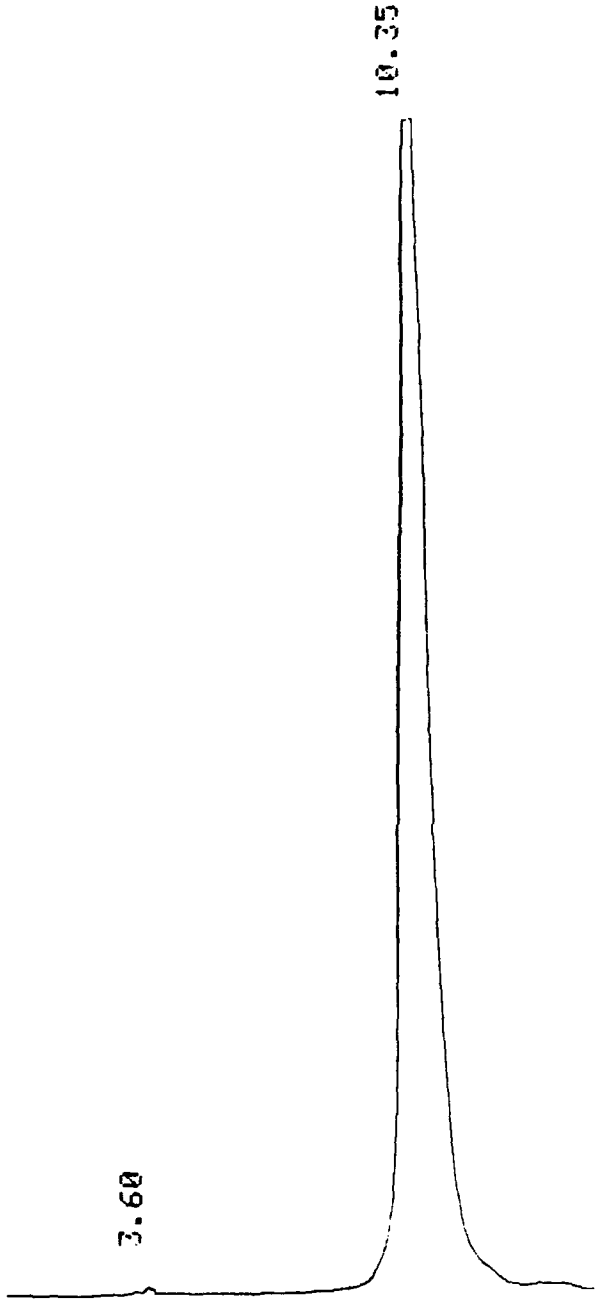

Figure 10. HPLC profile of PDGF B (115-128).
Solvent: Isocratic 13% CH_3CN + 0.1% TFA in H_2O;
Flowrate = 1 ml/min; Chart speed = 0.5 cm/min; UV = 220 nm.

Table 4
AMINO ACID COMPOSITION OF SYNTHETIC PEPTIDES

	Level[a] (mol/mol) in Peptide										
	PDGF A Chain Peptides			PDGF B Chain Peptides							
Amino Acid	12-28	44-51	101-125	1-20	22-36	45-52	55-67	86-114	101-117	104-116	115-128
Asp	0.99 (1)	0.92 (1)	1.9 (2)		2.99 (3)		1.92 (2)	3.12 (3)			
Thr	2.90 (3)	3.79 (2)	2.07 (2)	4.08 (4)	1.0 (1)		1.02 (1)	3.95 (4)	1.92 (2)	0.97 (1)	3.0 (3)
Ser	1.70 (2)	2.0 (2)	0.91 (1)	1.86 (2)	0.83 (1)	0.91 (1)		1.05 (1)	1.71 (2)	1.77 (2)	0.89 (1)
Glu	2.00 (2)		2.97 (3)	2.12 (2)	1.0 (1)	1.95 (2)	3.05 (3)	2.05 (2)	1.97 (2)	1.1 (1)	3.96 (4)
Pro	2.00 (2)		1.96 (2)	0.96 (1)			1.08 (1)	2 20 (2)	2.0 (2)	2.26 (2)	1.0 (1)
Gly		0.99 (1)	1.90 (2)	1.00 (1)		1.01 (1)			1.92 (2)	2.15 (2)	
Ala				3.19 (3)	0.99 (1)			5.18 (5)	2.93 (3)	2.15 (2)	0.95 (1)
Cys		ND				ND	ND				
Val	2.1 (2)				1.07 (1)	0.97 (1)	1.90 (2)	2.93 (3)	2.15 (2)	1.19 (1)	1.08 (1)
Ile	1.83 (2)			0.75 (1)	1.9 (2)						
Leu			1.07 (1)	1.93 (2)	1.0 (1)		1.06 (1)	2.08 (2)			
Tyr	0.99 (1)		1.0 (1)								
Phe					0.97 (1)						
Lys			5.08 (5)	1.01 (1)				3.17 (3)			0.97 (1)
His											
Arg	2.0 (2)		6.08 (6)	0.99 (1)	3.0 (3)	1.08 (1)	2.08 (2)	2.30 (2)	2.0 (2)	2.21 (2)	2.0 (2)
Trp											
Met				0.78 (1)							

[a]Values in parentheses indicate the expected number of amino acids in synthetic peptides. Values for Ser and Thr were corrected for 10% or 15% destruction during 24-hr hydrolysis. Quantities <0.1 mol/mol have been omitted.

ND = not determined.

To start the assay, we removed the binding buffer and added 0.25 mL of various concentrations of competitor (PDGF or peptides) to triplicate wells. ^{125}I-PDGF (5 ng) was added to each well in a volume of 0.25 mL, and binding was conducted typically for 2 hr at 4°C. The binding medium was aspirated, and the monolayers were washed 4× with 1 mL cold binding buffer at 4°C to remove the unbound radioactivity. The cells were solubilized in 0.5 mL of 1 N NaOH, and a portion of the lystate (0.4 mL) was transferred to tubes. The bound ^{125}I-PDGF was quantitated by using a gamma counter.

MITOGENESIS ASSAY

Balb/c 3T3 cells were set up at 20,000 cells per well in flat-bottom 96-well plates on day 0. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). On day 3, the medium was removed and the monolayers were washed once with DMEMo. Cells were incubated in DMEMo for 48 hr at 37°C. PDGF or peptides were added in DMEMo, and (^{3}H)-thymidine (^{3}H-TdR) was added (final concentration, 1 μCi/mL). The cells were incubated with (^{3}H-TdR) for 24 hr, then washed with PBS, trypsinized, and collected with an automated cell harvester onto glass fiber filter paper with 5% (w/v) trichloroacetic acid (TCA). The filters were dried, transferred to counting vials, and counted in a liquid scintillation counter. (^{3}H-TdR) incorporation was quantitated in 6 replicate wells.

CHEMOTAXIS ASSAY

The chemotactic response of Balb/c 3T3 cells to PDGF and peptides was assayed by using a modified Boyden chamber. Chemotaxis was quantitated by using a spectrophotometric assay (Grotendorst, 1987). Chambers were assembled by using polyvinylpyrrolidone-free polycarbonate filters (Nucleopore Corp.). The filters (8-μm-diameter pores) were prewetted in DMEMo containing 0.5% BSA. The lower wells contained 0.2 mL of various concentrations of PDGF or peptides in DMEMo + 0.5% BSA. The upper wells contained 100,000 3T3 cells in 0.2 mL. The assembled chambers were typically incubated for 16 hr at 37°C in 10% CO_2. After overnight incubation, the filters were removed from the chamber, fixed in methanol, and stained with Wright-Giemsa stain solution (Polysciences, Inc.). The stained filters were placed on glass microscope slides, and the upper surface cells were carefully removed by scraping with a cotton swab. Care was taken at this step to remove all cellular debris that contained stained material. After a small amount of water was added to each filter, the filters were removed from the slides, blotted on absorbant paper, and placed in 12- × 75-cm tubes containing 0.5 mL of 0.1 N HCl.

The tube was vortexed, and 0.2 mL was transferred to a 96-well plate for reading in a plate reader. The amount of stain measured at 650 nm for each condition was evaluated in replicates of four filters.

RESULTS AND DISCUSSION

BINDING OF PEPTIDES TO 3T3 FIBROBLASTS

During the year, we tested 13 peptides for cell binding; 3T3 fibroblasts which have receptors for both PDGF A and B chains, were used. Radiolabeled ^{125}I-PDGF BB was allowed to bind separately to 3T3 cells in the presence of increasing concentrations of unlabeled peptides to determine whether binding is mediated through the PDGF receptors or through some independent moiety. The results are shown in Table 5. Three peptides, PDGF A (44-51) and (101-125) and PDGF B (45-52) cyclic, competed with the binding of PDGF BB (Figures 11 through 13). PDGF A (101-125) was a weak inhibitor (Figure 11), but the IC_{50} of PDGF A (44-51) and B (45-52) cyclic were 443 and 18 nm, respectively. The cyclic analog of PDGF A (44-51) and the linear analog of PDGF B (45-52) did not inhibit the binding of ^{125}I-PDGF BB. These data suggest that both PDGF BB and the peptides bound to a common site. Moreover, the greater concentrations of peptides required to displace PDGF indicate that PDGF binds to 3T3 cells with greater affinity than do the peptides. The IC_{50} of PDGF BB is 4 pg (Figure 14). The inhibitory activities of peptides against PDGF AA and AB need to be tested.

Table 5
RESULTS OF THE COMPETITIVE BINDING ASSAY

Peptide	Concentration (ng or µg/well)	Inhibition (%)
^{125}I PDGF	5 ng	0
PDGF BB	100 ng	55
	50 ng	48
	25 ng	35
	12.5 ng	27
	6.25 ng	14
PDGF A (12-28)	500 µg	7
	250 µg	4
	125 µg	8
PDGF A (44-51)	500 µg	57
	250 µg	39
	125 µg	24
PDGF A (44-51) cyclic	500 µg	0
	250 µg	0
	125 µg	0
PDGF A (101-125)	500 µg	35
	250 µg	35
	125 µg	25
PDGF B (1-20)	500 µg	0
	250 µg	0
	125 µg	0
PDGF B (22-36)	500 µg	11
	250 µg	12
	125 µg	6
PDGF B (45-52)	500 µg	2
	250 µg	1
	125 µg	-3
PDGF B (45-52) cyclic	500 µg	71
	250 µg	71
	125 µg	70
	31.25 µg	64
	7.81 µg	13
PDGF B (55-67)	500 µg	-2
	250 µg	-10
	125 µg	6
PDGF B (86-114)	500 µg	6
	250 µg	0
	125 µg	1
PDGF B (101-117)	500 µg	8
	250 µg	10
	125 µg	10
PDGF B (104-116)	500 µg	-1
	250 µg	-2
	125 µg	-4
PDGF B (115-128)	500 µg	-2
	250 µg	-5
	125 µg	-7

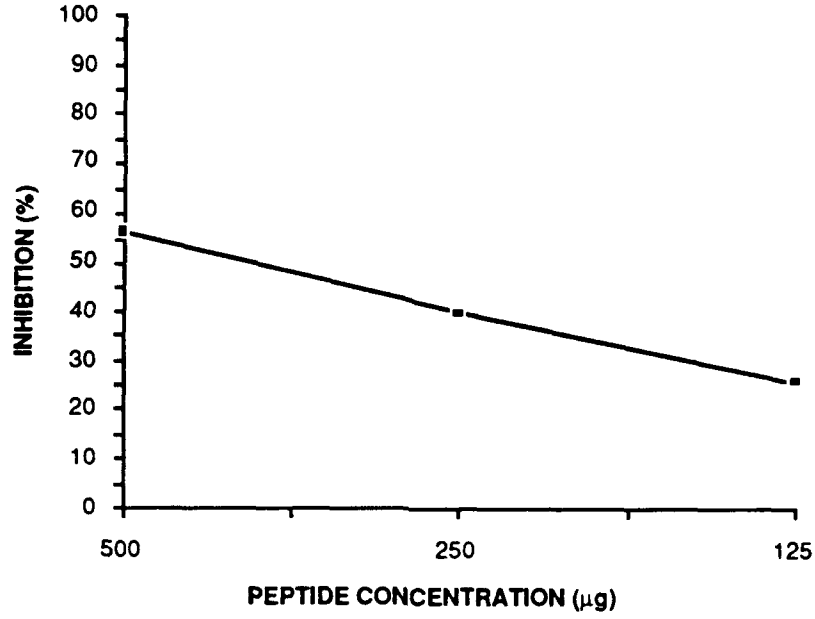

Figure 11. Competitive inhibition for receptor binding on 3T3 cells by PDGF A (44-51).

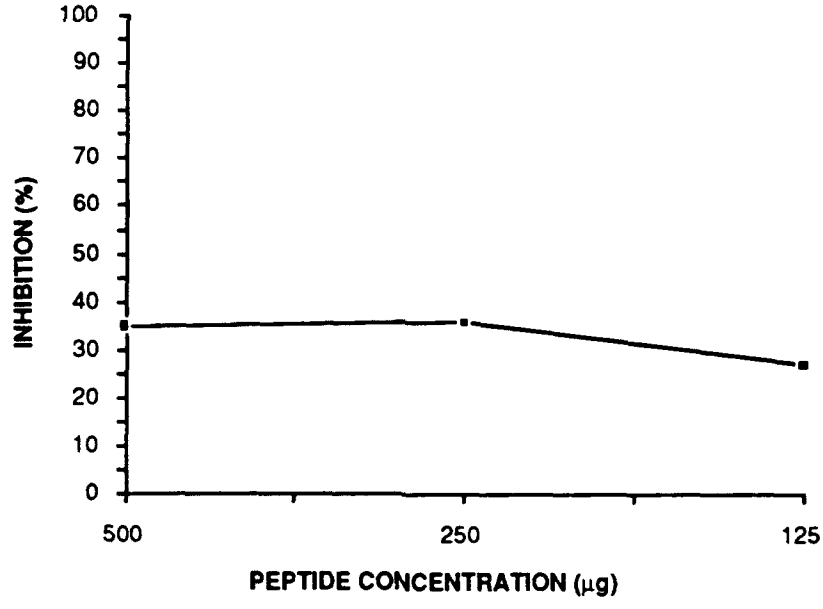

Figure 12. Competitive inhibition for receptor binding on 3T3 cells by PDGF A (101-125).

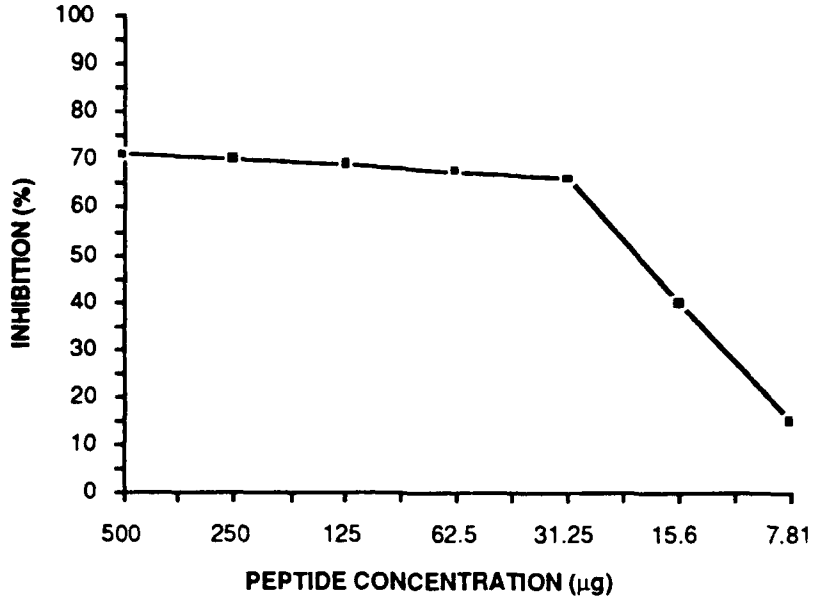

Figure 13. Competitive inhibition for receptor binding on 3T3 cells by PDGF B (45-52) cyclic.

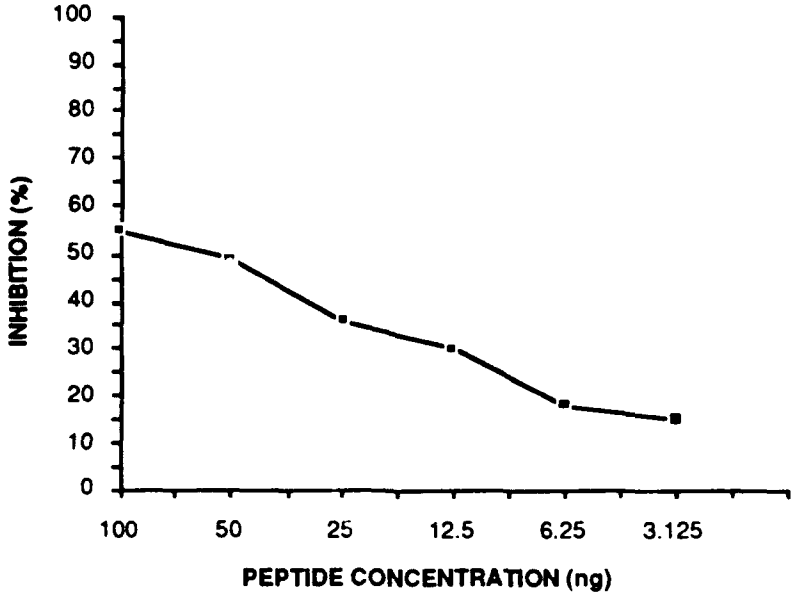

Figure 14. Competitive inhibition for receptor binding on 3T3 cells by PDGF.

EFFECT OF PEPTIDES ON DNA SYNTHESIS STIMULATED BY SERUM

Varying concentrations of each peptide were diluted in medium without serum and incubated with the cells to determine whether the peptides could modulate the level of DNA synthesis stimulated by serum. None of the peptides tested so far had any effect on DNA synthesis (Table 6). Recently, we investigated the effect of different serum concentrations in this assay. Results will be reported in our next quarterly report.

Table 6
THYMIDINE INCORPORATION AT PEPTIDE CONCENTRATION (cpm/well)

Peptide	Control (media)	1000 µg/mL	500 µg/mL	250 µg/mL
PDGF A (12-28)	8988 ± 1252	7238 ± 1064	11062 ± 2845	8327 ± 2144
PDGF A (44-51)	913 ± 414	242 ± 153	528 ± 468	616 ± 386
PDGF A (44-51) cyclic	718 ± 97	650 ± 161	663 ± 140	743 ± 249
PDGF A (101-125)	11798 ± 2863	5425 ± 610	12077 ± 1180	7593 ± 1771
PDGF B (1-20)	420 ± 151	346 ± 53	288 ± 172	378 ± 65
PDGF B (22-36)	7149 ± 1363	6709 ± 1396	8017 ± 1866	8093 ± 2491
PDGF B (45-52)	11402 ± 1275	12503 ± 3240	12527 ± 1853	10263 ± 1844
PDGF B (45-52) cyclic	1020 ± 375	751 ± 53	861 ± 235	1077 ± 537
PDGF B (55-67)	12141 ± 2308	13784 ± 1404	13921 ± 1918	11509 ± 2068
PDGF B (86-114)	11922 ± 1924	5829 ± 892	10798 ± 602	10992 ± 1947
PDGF B (101-117)	10366 ± 1585	7326 ± 1790	11636 ± 2171	6103 ± 1078
PDGF B (104-116)	6984 ± 734	7040 ± 1191	8725 ± 1197	7061 ± 1711
PDGF B (115-128)	12495 ± 1461	7943 ± 3047	13477 ± 928	7996 ± 1396

PDGF	100 ng	8082 ± 140
	50 ng	6497 ± 1099
	10 ng	1707 ± 246
	1 ng	646 ± 187
	Media	754 ± 373

EFFECT OF PEPTIDES ON CELL MIGRATION

Seven peptides were tested for their ability to stimulate cell migration. All three peptides that showed competitive binding were found to stimulate weak cell migratory or chemotactic activity. The data are shown in Table 7.

Table 7
CHEMOTACTIC ACTIVITY OF PEPTIDES

Peptide	Concentration (µg or ng/mL)	OD_{650}[a]	SD	Chemotactic Activity
Media		0.026	0.009	-
PDGF B (22-36)	250 µg	0.027	0.002	-
PDGF A (12-28)	250 µg	0.028	0.006	-
PDGF B (101-117)	250 µg	0.021	0.003	-
PDGF B (86-114)	250 µg	0.022	0.007	-
PDGF B (45-52) cyclic	250 µg	0.040	0.003	+
PDGF A (101-125)	250 µg	0.042	0.010	+
PDGF A (44-51)	250 µg	0.043	0.011	+
PDGF	100 ng	0.057	0.007	+
	50 ng	0.055	0.006	+
	20 ng	0.059	0.010	+
	10 ng	0.047	0.013	+
	1 ng	0.021	0.012	-
	0.5 ng	0.023	0.007	-

[a]*Peptides were considered positive when the OD was 1-1/2 times more than that of the media.*

PLANS FOR NEXT YEAR

We plan to synthesize the remaining peptides from Tables 3 through 6, pp.18 and 19; and Tables 9 and 10, p.23 of the original proposal. These peptides will be tested for cell binding, mitogenesis, and chemotaxis. Depending on the data obtained from the biological assays, we will initialize the *in vivo* wound-healing assay.

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